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Aberrant ETB receptor regulation of AT_1 receptors in immortalized renal proximal tubule cells of spontaneously hypertensive rats

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Aberrant ETB receptor regulation of AT_1 receptors in immortalized renal proximal tubule cells of spontaneously hypertensive rats.

Background. The renin-angiotensin and endothelin systems interact to regulate blood pressure, in part, by affecting sodium transport in the kidney. Because angiotensin II type 1 (AT_1) receptor activation increases ETB receptor expression in renal proximal tubule cells from Wistar-Kyoto (WKY) rat, we hypothesize that ETB receptor activation may also regulate AT_1 receptor expression. Furthermore, ETB receptor regulation of the AT_1 receptor may be different in the WKY and spontaneously hypertensive rat (SHR).

Method. AT_1 and ETB receptors were studied in immortalized renal proximal tubule cells from WKY and SHRs, using immunoblotting, confocal microscopic colocalization, and immunoprecipitation.

Results. In WKY renal proximal tubule cells, an ETB receptor agonist, BQ3020, decreased AT_1 receptor protein in a time- and concentration-dependent manner [median effective concentration (EC_{50}) $= 3.2 \times 10^{-10}$ mol/L, $t_{1/2} = 15$ hours]. The inhibitory effect of BQ3020 (10^{-8} mol/L/24 hours) on AT_1 receptor protein was blocked by an ETB receptor antagonist (BQ788). However, BQ3020 (10^{-8} mol/L/24 hours) increased ETB receptor protein in WKY renal proximal tubule cells. In contrast, in SHR renal proximal tubule cells, BQ3020 (10^{-8} mol/L/24 hours) no longer affected AT_1 or ETB receptor protein. AT_1/ETB receptors colocalized and coimmunoprecipitated in WKY and SHRs. BQ3020 (10^{-8} mol/L/15 minutes) treatment had no effect on AT_1/ETB coimmunoprecipitation in WKY but decreased it in SHRs. BQ3020 (10^{-8} mol/L/15 minutes) treatment increased AT_1 receptor phosphorylation in WKY, but decreased it in SHRs.

Conclusion. ETB receptors regulate AT_1 receptors by direct physical receptor interaction and receptor expression. An impaired ETB receptor regulation of the AT_1 receptor may participate in the pathogenesis of high blood pressure in the SHR.

Key words: essential hypertension, angiotensin II receptors, endothelin, kidney, phosphorylation.

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Enhanced sodium transport in the renal proximal tubule and thick ascending limb of Henle occurs in human essential and rodent genetic hypertension [1–3]. This enhanced sodium transport may be caused by increased activity of antinatriuretic agents and/or decreased activity of natriuretic agents [4–9].

The renin-angiotensin system plays a critical role in the regulation of blood pressure and renal excretory function [7–9]. Angiotensin II is functionally the most relevant peptide of this system, and its antinatriuretic and prohypertensive actions are mediated by the activation of the angiotensin II type 1 (AT_1) receptor [6–9].

Endothelins are a family of three isopeptides (ET1, ET2, and ET3), for which at least two types of receptors have been identified (ETA and ETB) [10, 11]. Renal proximal tubules express AT_1, ETA, and ETB receptors [7–11]. Several studies have shown that the ETA receptor decreases while the ETB receptor increases sodium excretion [12–17]. The interplay between the natriuretic ETB/antinatriuretic ETA and AT_1 receptors represents one pathway in the regulation of renal sodium and water excretion [18–20].

The renin-angiotensin system may exert some of its effects via an interaction with the endothelin system [18–20]. Angiotensin II regulates endothelin synthesis in the kidney [19]. Endothelin has multifaceted effects on the renin-angiotensin-aldosterone system, such as a dose-dependent inhibition of renin production and direct stimulation of aldosterone and promotion of growth of the adrenal cortex [18–21].

In a preliminary communication, we showed that AT_1 and ETB receptors colocalize and coimmunoprecipitate in renal proximal tubule cells, and stimulation of the AT_1 receptor increases ETB receptor expression in Wistar-Kyoto (WKY) rats [abstract; Zeng C, et al, *Hypertension* 26:80A, 2003]. We hypothesize that the ETB receptor may also regulate the AT_1 receptor, including its expression. In the current study we examined the interaction between AT_1 and ETB receptors in immortalized rat renal proximal tubule cells, which have characteristics similar to freshly obtained renal proximal tubule brush border membranes and renal proximal tubules, at least with regard to D_1 receptors and their responses to G protein stimulation [22–25]. We now report that long-term activation of the ETB receptor decreases AT_1 receptor and increases ETB receptor expression in renal proximal tubule cells from WKY rats. In contrast, in the spontaneously hypertensive rat (SHR), the ETB receptor has no effect on AT_1 receptor expression. ETB receptor activation does not influence AT_1 and ETB receptor coimmunoprecipitation in WKY cells, but decreases it in SHR cells. Short-term treatment with the ETB agonist, BQ3020, increases phosphorylation of the AT_1 receptor in WKY cells, but decreases it in SHR cells. We conclude that ETB receptors regulate AT_1 receptors by direct physical receptor interaction and receptor expression. The role of ETB regulation of AT_1 receptor expression and phosphorylation on renal proximal tubule ion transport, however, remains to be determined.

METHODS

Cell culture

Immortalized renal proximal tubule cells from 4- to 8-week-old SHR and WKY rats were cultured at 37°C in 95% air/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/F-12 culture media, as previously described [abstract; Zeng C, et al, *Hypertension* 26:80A, 2003] [22, 25–27]. The cells (80% confluence) were extracted in ice-cold lysis buffer [phosphate-buffered saline (PBS) with 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L ethyleneglycol tetraacetate (EGTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, and 10 μg/mL leupeptin), sonicated, placed on ice for 1 hour, and centrifuged at 16,000*g* for 30 minutes. The supernatants were stored at −70°C until use for immunoblotting and/or immunoprecipitation.

Immunoblotting

The amino acid sequence of the AT_1 receptor immunogenic peptide (rabbit antihuman AT_1 receptor antibody) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) is QDDCPKAGRHC (amino acids 15 to 24) [22, 27]. The amino acid sequence of the ETB receptor immunogenic peptide (rabbit antirat ETB receptor antibody) (Alomone Labs, Jerusalem, Israel) is CEMLRKKSGMQIALND (amino acids 298 to 314) [28]. The specificities of these antibodies have been reported [22, 27, 28]. Renal proximal tubule cells were treated with vehicle (dH_2O), an ETB agonist (BQ3020) [29, 30] (Sigma Chemical Co., St. Louis, MO, USA), and/or an ETB receptor antagonist (BQ788) [29, 30] (Sigma Chemical Co.), at the indicated concentrations and times. BQ3020 is 1000-fold selective to the ETB receptor relative to the ETA receptor in human kidney and other tissues [31–33]. Immunoblotting was performed as reported [22–27], except that the transblots were probed with ETB (1:300) or AT_1 receptor antibodies (1:400).

Confocal microscopy of doubly stained renal proximal tubule cells

Renal proximal tubule cells grown on coverslips were fixed and permeabilized with 100% methanol (for 30 minutes) [34, 35]. The ETB receptor was visualized by a rabbit antirat ETB receptor antibody followed by a fluorescein isothiocyanate (FITC)-conjugated antirabbit secondary antibody (Molecular Probes, Eugene, OR, USA). The AT_1 receptor was visualized by a mouse anti-AT_1 receptor monoclonal antibody (Abcam Limited, Cambridgeshire, UK), followed by an Alexa Fluor 568-goat antimouse IgG antibody (Molecular Probes). Cells on coverslips were mounted with the ProLong Antifade Kit (Molecular Probes). Negative controls included absence of the primary or the secondary antibodies, or antibodies preadsorbed with the immunizing peptide (1:10 wt/wt). The immunofluorescence densities and images were acquired (Olympus AX70) at an excitation wavelength of 488 nm and 568 nm; emission was detected at 535 and 645 nm.

Immunoprecipitation

Renal proximal tubule cells were incubated with vehicle (dH_2O) or BQ3020 (10^{-8} mol/L) for 15 minutes, as described above. The cells were lysed with ice-cold lysis buffer for 1 hour and centrifuged at 16,000*g* for 30 minutes. Equal amounts of lysates (500 μg protein/mL supernatant for renal proximal tubule cells from WKY rats and SHRs) were incubated with affinity-purified anti-ETB receptor antibody (for AT_1/ETB receptor coimmunoprecipitation) or polyclonal antiphosphoserine antibody (Zymed Laboratory, South San Francisco, CA, USA) (for AT_1 receptor phosphorylation) (1 μg/mL) for 1 hour and protein G agarose at 4°C for 12 hours. The immunoprecipitates were pelleted and washed four times with lysis buffer. The pellets were suspended in sample buffer, boiled for 10 minutes, and subjected to immunoblotting with the AT_1 receptor. In order to determine the specificity of the bands, normal rabbit IgG (negative control)

and AT_1 receptor antibody (positive control) were used as immunoprecipitants. The density of the bands were semiquantified by densitometry using Quantiscan (Ferguson, MO, USA), as previously reported [22–27].

Statistical analysis

The data are expressed as mean ± SEM. Comparison within groups was made by repeated-measures analysis of variance (ANOVA) and comparison among groups was made by factorial ANOVA and Holm-Sidak test. A value of $P < 0.05$ was considered significant.

RESULTS

ETB receptors decrease AT_1 receptor expression in renal proximal tubule cells from WKY rats, but not from SHRs

An ETB receptor agonist, BQ3020, decreased AT_1 receptor expression in a concentration- and time-dependent manner in renal proximal tubule cells from WKY rats. The inhibitory effect was evident at 10^{-10} mol/L with a 50% decrease [median effective concentration (EC_{50})] at 3.2×10^{-10} mol/L (Fig. 1A). The inhibitory effect of BQ3020 (10^{-8} mol/L) was noted as early as 2 hours and maintained for at least 30 hours; a 50% decrease ($t_{1/2}$) in AT_1 receptor expression occurred at 15 hours (Fig. 1B). In renal proximal tubule cells from SHRs, BQ3020 had no effect on AT_1 receptor expression [SHR control = 1.08 ± 0.08, BQ3020 (10^{-8} mol/L/24 hours) = 1.04 ± 0.12] ($N = 15$) [WKY control = 1.12 ± 0.12, BQ3020 (10^{-8} mol/L/24 hours) = 0.76 ± 0.08] ($N = 15$) (Fig. 1C).

To determine whether higher concentrations of BQ3020 could have an effect on AT_1 receptor expression, SHR cells were treated with varying concentrations of BQ3020 for 24 hours. Consistent with the results from Figure 1C, 10^{-11} to 10^{-7}M BQ3020 had no effect on AT_1 receptor protein expression (Fig. 1D).

The specificity of BQ3020 as an ETB receptor agonist was also determined by studying the effect of the ETB receptor antagonist, BQ788. Consistent with the studies shown in Figure 1A to C, BQ3020 (10^{-8} mol/L/24 hours) decreased AT_1 receptor expression (control = 1.08 ± 0.04, BQ3020 = 0.76 ± 0.04) ($N = 7$) ($P < 0.05$). The ETB receptor antagonist, BQ788 (10^{-8} mol/L), by itself, had no effect on AT_1 receptor expression (1.08 ± 0.08), but reversed the inhibitory effect of BQ3020 on ETB receptor expression (1.09 ± 0.06) (Fig. 1E).

BQ3020 increases ETB receptor expression in renal proximal tubule cells from WKY rats, but not from SHRs

To investigate the effect of BQ3020 on ETB receptors, renal proximal tubule cells were incubated with BQ3020 (10^{-8} mol/L/24 hours) at the indicated times and concentrations. BQ3020 increased ETB receptor expression in renal proximal tubule cells from WKY rats (control = 0.92 ± 0.12, BQ3020 = 1.24 ± 0.12) ($P < 0.05$) ($N = 11$). However, there was no effect in SHR cells (control = 1.00 ± 0.08, BQ3020 = 0.88 ± 0.16) ($N = 11$) (Fig. 2).

ETB receptor colocalizes and coimmunoprecipitates with the AT_1 receptor in rat renal proximal tubule cells

In order to determine the potential for a direct or an indirect interaction between ETB and AT_1 receptors, we studied the colocalization of ETB and AT_1 receptors in renal proximal tubule cells from WKY rats by confocal microscopy. Both ETB and AT_1 receptors were found throughout the cell with evidence of colocalization, especially at the cell surface membrane (Fig. 3).

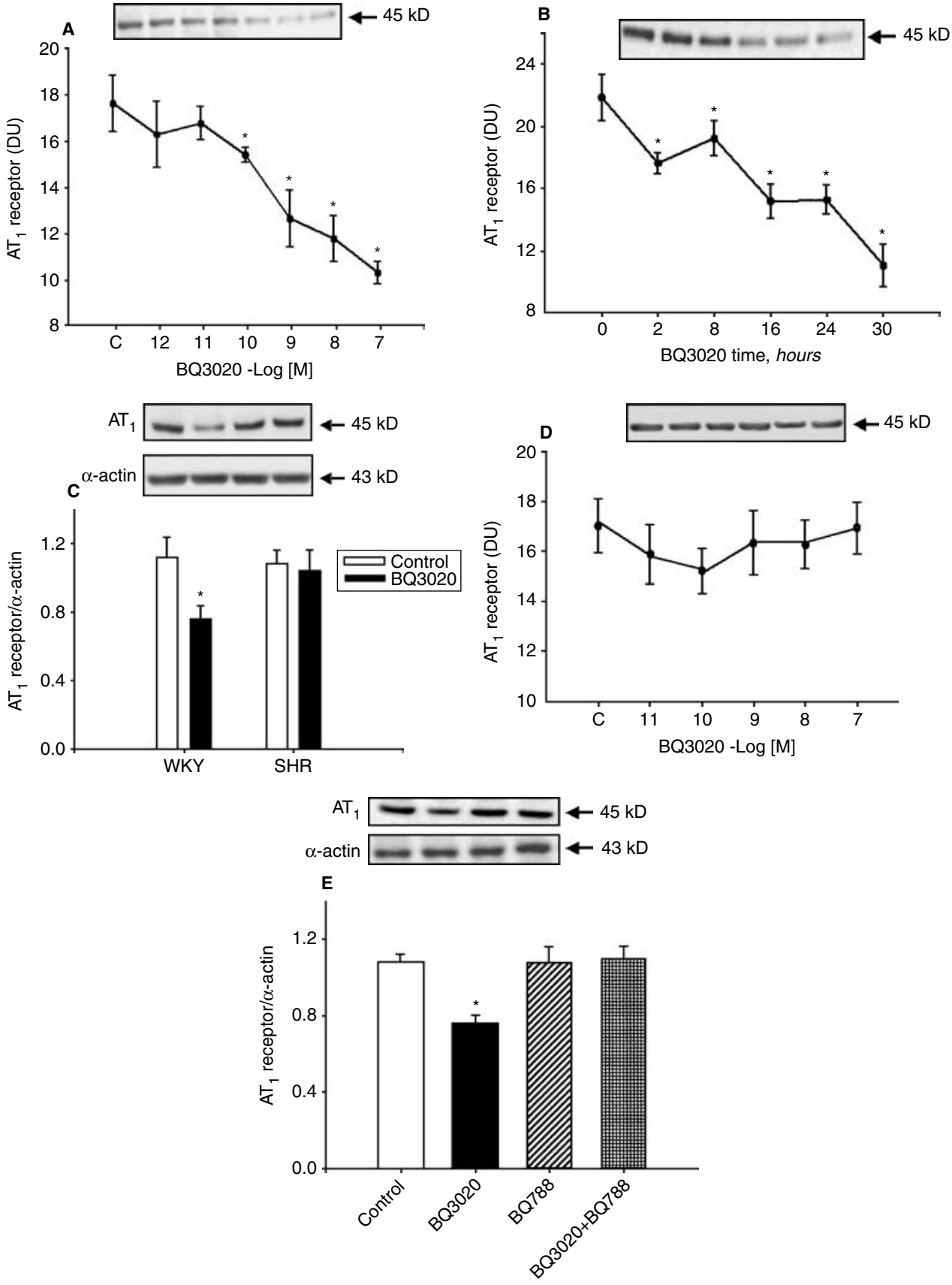
To determine whether there is a physical interaction between the ETB and the AT_1 receptor, additional experiments were performed. ETB receptors were first immunoprecipitated with anti-ETB receptor antibodies and then probed with anti-AT_1 receptor antibodies. As shown in Figure 4, the 45 kD band representing the coimmunoprecipitated ETB and AT_1 receptors was not changed by a 15-minute treatment of BQ3020 (10^{-8} mol/L) in renal proximal tubule cells from WKY rats [control = 27 ± 2 density units (DU), BQ3020 = 28 ± 1 DU] ($N = 8$). However, BQ3020 decreased ETB and AT_1 receptor coimmunoprecipitation in SHR cells (control = 28 ± 1 DU, BQ3020 = 17 ± 2 DU) ($N = 8$) ($P < 0.05$).

BQ3020 increases AT_1 receptor phosphorylation in WKY cells, but decreases it in SHR cells

The AT_1 receptor has cytoplasmic carboxyl-terminal tail domains that are rich in serine and threonine residues; phosphorylation of these residues impairs AT_1 receptor function [36]. To investigate the effect of BQ3020 on AT_1 receptor phosphorylation, renal proximal tubule cells were treated for 15 minutes. As shown in Figure 5, BQ3020 increased AT_1 receptor phosphorylation in WKY cells, but decreased it in SHR cells (WKY control = 23 ± 3 DU, BQ3020 = 34 ± 2 DU; SHR control = 26 ± 3 DU, BQ3020 = 17 ± 2 DU) ($N = 9$) ($P < 0.05$).

DISCUSSION

The renin-angiotensin-aldosterone system and the endothelin system are two important systems that regulate blood pressure and renal sodium excretion. Recent studies indicate that angiotensin II may exert some of its effects via an interaction with the endothelin system. Thus, angiotensin II has been reported to increase endothelin-1 synthesis in the kidney [19] and expression of endothelin-converting enzyme-1 in human umbilical vein endothelial cells [37]. The hypertrophic and mitogenic effects of angiotensin II may also be augmented by endothelin, via


A
45 kD
AT1 receptor (DU)
BQ3020 -Log [M]
C 12 11 10 9 8 7
B
45 kD
AT1 receptor (DU)
BQ3020 time, hours
0 2 8 16 24 30
AT1
45 kD
α-actin
43 kD
C
AT1 receptor/α-actin
Control
BQ3020
WKY
SHR
D
45 kD
AT1 receptor (DU)
BQ3020 -Log [M]
C 11 10 9 8 7
AT1
45 kD
α-actin
43 kD
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BQ788
BQ3020+BQ788

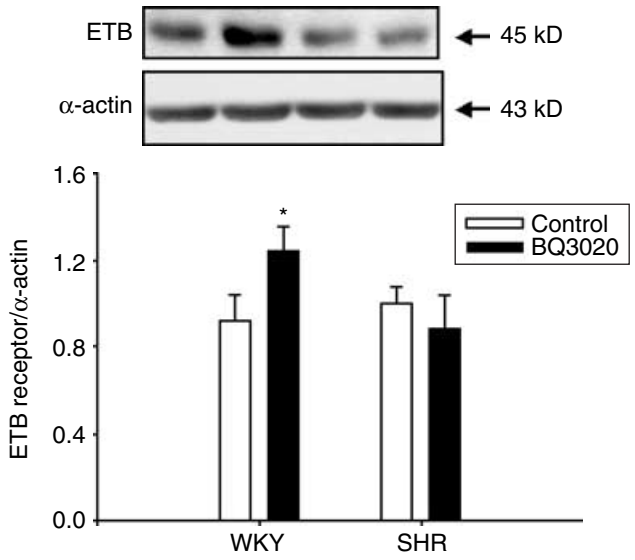

Fig. 2. Differential effects of BQ3020 (10^{-8} mol/L/24 hours) on ETB receptor expression in renal proximal tubule cells from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). The cells were incubated with the indicated time and concentration. Results are expressed as the ratio of angiotensin II type 1 (AT_1) receptor and α-actin densities ($N = 11$). $^*P < 0.05$ vs. control, ANOVA, Holm-Sidak test.

ETA receptors [38]. Endothelin, via ETA receptors, may also act as an amplifier of the vasoconstrictor effect of angiotensin [39]. There is reciprocal regulation between the two systems. Endothelin-1 has been reported to decrease renin secretion [21]. Endothelin may regulate not only the hemodynamic effects of angiotensin II but also the pathologic consequences of increased activity of the renin-angiotensin-aldosterone system. For example, the hypertension associated with chronic angiotensin II infusion can be attenuated by an ETA/ETB receptor antagonist [19, 20]. In a canine model of Page (kidney wrapping) hypertension, bosentan, a combined ETA and ETB receptor antagonist, exerts a hypotensive effect in addition to that caused by the AT_1 receptor antagonist losartan [40]. However, there are no reports on the ability of ETB receptors to regulate AT_1 receptor expression.

We have reported that ETB and AT_1 receptors interact in rat renal proximal tubule cells [abstract; Zeng C, et al, *Hypertension* 26:80A, 2003]. Our current studies support our previous report that ETB and AT_1 receptors can regulate each other at the protein and cell biologic level [abstract; Zeng C, et al, *Hypertension* 26:80A, 2003]. The interaction among G protein–coupled receptors can be synergistic, additive, or negative. The regulation may occur at the physiologic level where one functional effect antagonizes the other. In the case of the AT_1 receptor, its vasoconstrictor effect can augment the ability of ETA and ETB to contract vascular smooth muscle cells [41, 42]. The increase in renal tubular sodium transport caused by AT_1 receptors can augment a similar effect of ETA receptors. However, the ability of ETB receptors to stimulate vasodilatory agents from endothelial cells would be counter regulatory [43–46], as would be the ability of renal tubular ETB receptors to decrease sodium transport [10–13, 18].

The ETB receptor has been shown to decrease sodium transport in the renal medullary collecting duct and medullary thick ascending limb of Henle [10–13, 18]. However, both inhibitory and stimulatory effects of endothelin have been reported in the proximal tubule [16, 47–49]. In the rat, endothelin acutely inhibits fluid and bicarbonate transport by reducing Na^+/K^+ adenosine triphosphatase (ATPase) activity [17]. Short-term stimulation of ETB receptors in opossum kidney cells, a renal proximal tubular cell line, activates the sodium hydrogen exchanger, NHE3 [50]. However, chronic treatment of the same opossum kidney cells by endothelin has an opposite effect on NHE3 activity [15]. Thus, a 6-hour exposure of opossum kidney cells to endothelin-1 inhibits NHE3 expression and activity [15]. It is of interest that the ability of an ETB receptor agonist to decrease AT_1 receptor expression also occurs within the same time frame as the ability of ETB to inhibit NHE3 expression and activity. Another G protein–coupled receptor, the D_3 dopamine receptor has been shown to inhibit NHE3 activity in rat renal proximal tubules and immortalized rat renal proximal tubule cells [51]. We have preliminary data

Fig. 1. Effect of BQ3020 on angiotensin II type 1 (AT_1) receptor expression in renal proximal tubule cells from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). (*A*) Concentration-response of AT_1 receptor expression in renal proximal tubule cells from WKY rats treated with the BQ3020. Immunoreactive AT_1 receptor expression was determined after a 24-hour incubation with the indicated concentrations of BQ3020. Results are expressed as density units (DU) ($N = 9$). $^*P < 0.05$ vs. control, analysis of variance (ANOVA), Holm-Sidak test. (*B*) Time-course of AT_1 receptor expression in renal proximal tubule cells from WKY rats treated with BQ3020. The cells were incubated for the indicated times with 10^{-8} mol/L BQ3020. Results are expressed as DU ($N = 10$). $^*P < 0.05$ vs. control (0 = time), ANOVA, Holm-Sidak test. (*C*) Differential effects of BQ3020 (10^{-8} mol/L/24 hours) on AT_1 receptor expression in renal proximal tubule cells from both WKY rats and SHRs. The cells were incubated with the indicated time and concentration. Results are expressed as the ratio of AT_1 receptor and α-actin densities ($N = 15$). $^*P < 0.05$ vs. control, ANOVA, Holm-Sidak test. (*D*) Concentration-response of AT_1 receptor expression in SHR renal proximal tubule cells treated with the BQ3020. Immunoreactive AT_1 receptor expression was determined after a 24-hour incubation with the indicated concentrations of BQ3020. Results are expressed as DU ($N = 6$) (P = NS vs. control, ANOVA, Holm-Sidak test). (*E*) Effect of BQ3020 and an ETB antagonist (BQ788) on AT_1 receptor expression in renal proximal tubule cells from WKY rats. The cells were incubated with the indicated reagents (BQ3020, 10^{-8} mol/L; BQ788, 10^{-8} mol/L) for 24 hours. Results are expressed as the ratio of AT_1 receptor and α-actin densities ($N = 7$). $^*P < 0.05$ vs. others, ANOVA, Holm-Sidak test.

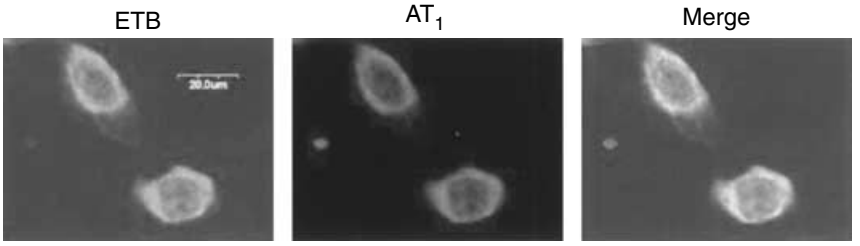

Fig. 3. ETB and angiotensin II type 1 (AT_1) receptor colocalization in renal proximal tubule cells from Wistar-Kyoto (WKY) rats. The cells were washed, fixed, and immunostained for ETB and AT_1 receptors, as described in the Methods section. Colocalization appears as yellow after merging the images of fluorescein isothiocyanate (FITC)-labeled ETB receptor (green) and Alexa 568-labeled AT_1 receptor (red).

showing that the acute (2 hours) natriuretic effect of a D_3 receptor agonist, PD128907, can be blocked by an ETB receptor antagonist, BQ788 [Zeng C, et al, unpublished data, 2004]. These preliminary results could be taken to indicate that the ETB receptor can regulate sodium transport in the renal proximal tubule, in vivo, by interacting with the D_3 receptor. These studies provide evidence for a potential interaction between the ETB and AT_1 receptor, in vivo. We now report that short-term (15 minutes) BQ3020 treatment increases AT_1 receptor phosphorylation in WKY renal proximal tubule cells. The ability of ETB receptors to decrease AT_1 receptor expression and to increase its phosphorylation [36] would be in keeping with a counterregulatory effect of ETB receptors on AT_1 receptor action on renal tubular sodium transport. The decrease in AT_1 receptor phosphorylation with BQ3020 in SHR renal proximal tubule cells could participate in the enhanced AT_1 receptor-mediated sodium reabsorption in hypertension. Direct proof of this action needs to be shown, however.

The mechanism of the decrease in AT_1 receptors caused by ETB receptors was not studied. However, we find that short-term stimulation of the ETB receptor increases AT_1 receptor phosphorylation in WKY cells; phosphorylation has been reported to prompt AT_{1A} receptor desensitization and internalization [36, 52]. The importance of G protein–coupled receptor kinase (GRK) in regulating G protein–coupled receptor function, including the AT_1 and ETB receptors, has been the subject of several reviews [53–55]. The AT_1 receptor is regulated by casein kinase 1α and GRK2 [54–56]. We have reported that GRK2, to a lesser extent, and GRK4, to a greater extent, regulates the D_1 dopamine receptor [57]. We have found that GRK4 serine phosphorylates and inactivates the D_1 dopamine receptor [58]. In a preliminary communication, we reported that there is a negative counter regulation of D_1 receptor and GRK4 expression [abstract; Felder RA, et al, *Hypertension* 42:438, 2003]. GRK4 may also regulate the ETB receptor because ETB receptor expression in renal cortex is higher and ETB receptor phosphorylation is lower in GRK4 A142V transgenic mice [Zeng C, et al, unpublished data, 2004]. It is possible that there is also negative regulation between ETB receptor and GRK4, and that the ETB receptor may regulate the AT_1 receptor via GRK4.

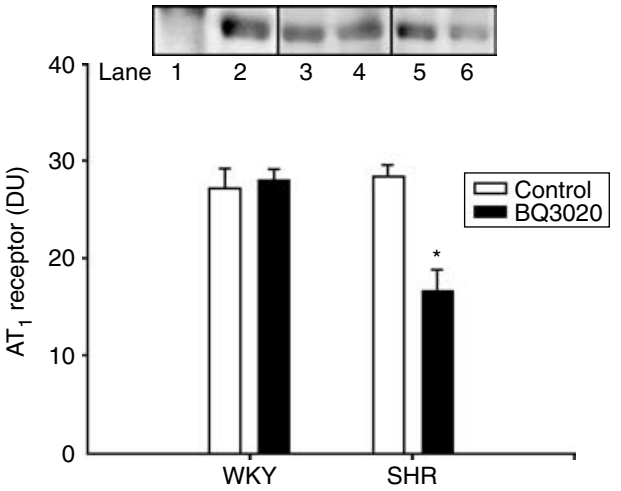

Fig. 4. Effect of BQ3020 on the coimmunoprecipitation of angiotensin II type 1 (AT_1) and ETB receptors in rat renal proximal tubule (RPT) cells. The cells were incubated with BQ3020 (10^{-8} mol/L) for 15 minutes. Thereafter, the samples were immunoprecipitated with anti-ETB receptor antibodies and immunoblotted with anti-AT_1 receptor antibodies. $^*P < 0.05$ vs. control ($N = 8$), analysis of variance (ANOVA), Holm-Sidak test. One immunoblot (45 kD) is depicted in the inset [lane 1, negative control; lane 2, positive control; lane 3, vehicle-treated renal proximal tubule cell of Wistar-Kyoto (WKY) rats; lane 4, BQ3020-treated renal proximal tubule cell of WKY rats; lane 5, vehicle-treated renal proximal tubule cell of spontaneously hypertensive rats (SHRs); and lane 6, BQ3020-treated renal proximal tubule cell of SHRs]. For a positive control anti-AT_1 antibodies (1 μg/mL) were used as the immunoprecipitant; for a negative control, normal rabbit IgG (1 μg/mL) was used as the immunoprecipitant instead of the anti-ETB antibodies and immunoblotted with anti-AT_1 antibodies as above.

The interaction between ETB and AT_1 receptors is rat strain dependent. Our previous study in renal proximal tubule cells indicates that long-term activation (hours) of the AT_1 receptor decreases AT_1 receptor expression and increases ETB receptor expression in WKY cells. In contrast, in SHRs, AT_1 receptor activation increases AT_1 receptor expression without affecting ETB receptor expression [abstract; Zeng C, et al, *Hypertension* 26:80A, 2003]. In this study, we find that long-term activation of the ETB receptor decreases AT_1 receptor expression and increases ETB receptor in WKY renal proximal tubule cells but has no effect on either ETB or AT_1 expression in SHRs. We now report that AT_1 and ETB receptors colocalize in WKY renal proximal tubule cells [abstract; Zeng C, et al, *Hypertension* 26:80A, 2003]. These two

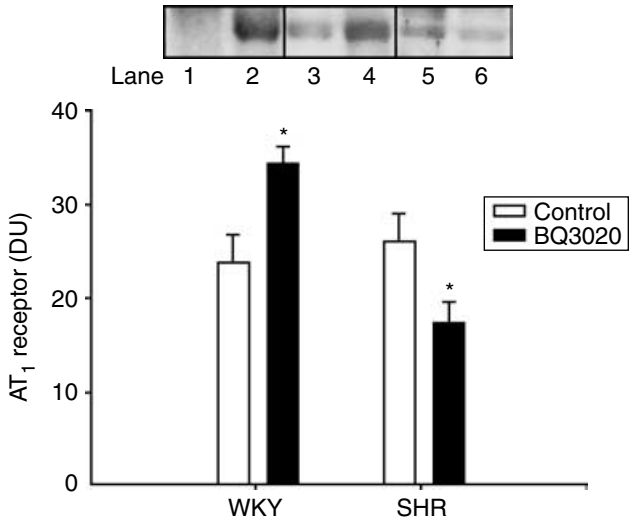

Fig. 5. Effect of BQ3020 on angiotensin II type 1 (AT_1) receptor phosphorylation in renal proximal tubule cells from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). The renal proximal tubule cellular lysate protein was immunoprecipitated with antiphosphoserine antibody and immunoblotted with anti-AT_1 receptor antibody as described in the **Methods** section. One immunoblot (45 kD) is depicted in the inset (lane 1, negative control; lane 2, positive control; lane 3, vehicle-treated renal proximal tubule cell of WKY rats; lane 4, BQ3020-treated renal proximal tubule cell of WKY rats; lane 5, vehicle-treated renal proximal tubule cell of SHRs; and lane 6, BQ3020-treated renal proximal tubule cell of SHRs]. $^*P < 0.05$ vs. control ($N = 9$), analysis of variance (ANOVA), Holm-Sidak test. For a positive control anti-AT_1 antibodies (1 μg/mL) were used as the immunoprecipitant; for a negative control, normal rabbit IgG (1 μg/mL) was used as the immunoprecipitant instead of the antiphosphoserine antibodies and immunoblotted with anti-AT_1 antibodies as above.

receptors directly interact with each other because they coimmunoprecipitate and activation of AT_1 receptor increases AT_1/ETB coimmunoprecipitation in WKY but not in SHR cells [abstract; Zeng C, et al, *Hypertension* 26:80A, 2003]. In the current study, ETB receptor activation does not affect AT_1 and ETB receptor coimmunoprecipitation in WKY but decreases it in SHR cells. We now also report that short-term (minutes) activation of ETB receptors increases the serine phosphorylation of the AT_1 receptor in WKY cells, but decreases it in SHR cells. The decrease in AT_1/ETB receptor coimmunoprecipitation in renal proximal tubularRPT cells following ETB receptor agonist stimulation could not have been caused by the changes in the expression of either ETB or AT_1 receptors, because the duration of stimulation is too short (15 minutes) to be caused by changes in receptor expression. However, the results in immortalized renal proximal tubule cells need to be confirmed in freshly isolated renal proximal tubule cells. Whereas the expression and transduction of the D_1 dopamine receptor signal is similar in freshly isolated and immortalized renal proximal tubule cells [22–25], this may not be the case for other G protein–coupled receptors (e.g., D_3 receptor [59]). Given this caveat, our studies suggest that the activation of the ETB receptor results in its modification or action on some adapter protein(s) that links AT_1 and ETB receptors. Moreover, this interaction is different between WKY rats and SHRs. It is possible that the dissociation of ETB receptors from AT_1 receptors after ETB receptor stimulation allows these receptors to exert their actions separately. The inability of ETB receptors to decrease AT_1 receptor expression in renal proximal tubule of SHRs and a failure to decrease ETB/AT_1 receptor heterodimerization could lead to increased AT_1 receptor function. The ETB receptor-induced decrease in the phosphorylation of the AT_1 receptor in SHR cells may also increase AT_1 receptor activity [36]. Renal tubular AT_1 receptor action is enhanced in SHRs [7, 60–62].

CONCLUSION

We have demonstrated that ETB receptors negatively regulate the expression of AT_1 receptors in renal proximal tubule cells from WKY rats but not in SHRs. Short-term activation of the ETB receptor increases AT_1 receptor phosphorylation in WKY cells but decreases it in SHR cells. We conclude that ETB receptors regulate AT_1 receptors by direct physical receptor interaction and receptor expression. The impaired natriuretic effect in SHRs may, in part, be related to impaired ETB receptor regulation of AT_1 receptors.

Perspectives

This study shows that long-term activation of ETB receptor decreases AT_1 receptor expression while short-term activation increases AT_1 receptor phosphorylation in WKY renal proximal tubule cells; these effects are not seen in SHR renal proximal tubule cells. AT_1 and ETB receptors may modulate each other to maintain a normal salt balance and a normotensive state. The loss of this counter regulation may be another mechanism for the sodium retention and the increase in blood pressure in SHRs.

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